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T Regulatory and Primed Uncommitted CD4 T Cells Express CD73, Which Suppresses Effector CD4 T Cells by Converting 5’-Adenosine Monophosphate to Adenosine

James J. Kobie, Pranav R. Shah, Li Yang, Jonathan A. Rebhahn, Deborah J. Fowell, and Tim R. Mosmann

CD73 (5’-ectonucleotidase) is expressed by two distinct mouse CD4 T cell populations: CD25+ (FoxP3+) T regulatory (Treg) cells that suppress T cell proliferation but do not secrete IL-2, and CD25− uncommitted primed precursor Th (Thpp) cells that secrete IL-2 but do not suppress in standard Treg suppressor assays. CD73 on both Treg and Thpp cells converted extracellular 5’-AMP to adenosine. Adenosine suppressed proliferation and cytokine secretion of Th1 and Th2 effector cells, even when target cells were activated by anti-CD3 and anti-CD28. This represents an additional suppressive mechanism of Treg cells and a previously unrecognized suppressive activity of Thpp cells. Infiltration of either Treg or Thpp cells at inflammatory sites could potentially convert 5’-AMP generated by neutrophils or dying cells into the anti-inflammatory mediator adenosine, thus dampening excessive immune reactions. The Journal of Immunology, 2006, 177: 6780–6786.

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Treatment of T lymphocytes with adenosine or the A2A agonist inhibits proliferation (13, 14), as well as the secretion of cytokines including IL-2, TNF-α, and IFN-γ (14, 15). However, the concentrations of adenosine required to elicit in vitro effects (25 μM) (13) were substantially higher than the Kd of various adenosine analogues for the A2A receptor (20–50 nM) (16). The Kd of the A2A receptor for adenosine itself is difficult to determine (17).

The generation of adenosine at sites of inflammation, hypoxia, organ injury, and traumatic shock is in part mediated by two sequential enzymes. The ecto-ATP diphosphohydrolase (CD39) expressed by neutrophils rapidly converts circulating ATP and ADP to 5’-AMP (18). CD73, expressed on the surface of endothelial cells (19) and a subset of T cells (20–22), is an ecto-5’-nucleotidase (23) that can convert 5’-AMP to adenosine.

We have recently identified, directly ex vivo, a population of memory CD4 T cells that express CD73 but not Ly-6A/E (22). These cells are similar to the uncommitted primed precursor helper cells (Thpp) previously identified in vitro (24). Both cell types secrete IL-2 and chemokines but not IFN-γ or IL-4 when activated (22, 24), both localize preferentially to lymph nodes (22, 25), and both have the flexibility to differentiate further into either Th1 or Th2 phenotypes depending on the polarizing cytokine environment. These Thpp cells may provide an expanded pool of uncommitted Ag-specific T cells without potentially harmful inflammatory functions.

The inhibitory activity of CD4 T cells has primarily been associated with FoxP3+ Treg cells, many of which express CD25 (26, 27). FoxP3 is important in the development and activity of Treg cells, because CD25+ cells isolated from FoxP3-deficient mice lack regulatory activity in vitro and in vivo, and retroviral expression of FoxP3 in nonregulatory T cells confers regulatory activity (28, 29). The mechanism(s) by which Tregs control immune responses in vivo and in vitro may include the suppressive cytokines TGF-β and IL-10, modulation of APC function, and sequestering of IL-2 (30).

3 Abbreviations used in this paper: Thpp, primed precursor Th cells; APCP, α,β-methylene ADP.
We now show that Treg cells also express CD73. Although Treg and Thpp cells also share other properties and both can differentiate from naive CD4 T cells under similar conditions in vitro, we show that these are two distinct populations. Adenosine produced by either cell type potently suppresses proliferation of effector CD4 T cells and also inhibits their production of cytokines. The inhibition of effector T cells occurs at low adenosine levels (provided that adenosine degradation is minimized), and either Treg or Thpp cells derived directly ex vivo can significantly inhibit effector T cells at a ratio of less than 1:1. Thus, the CD73/adenosine pathway is a potent additional suppressive pathway of Treg cells and also confers a suppressive anti-inflammatory function on the uncommitted precursor Thp cell type.

Materials and Methods

Mice

Female C57BL/6 and C57BL/6.PL mice (6–8 wk of age) were obtained from The Jackson Laboratory. All animal experiments were approved by the University of Rochester University Committee on Animal Resources (Rochester, NY).

Abs and cell lines

Purified anti-mouse IL-2 (clone JES6-1A12) and biotinylated anti-mouse IL-2 (clone JES6-5H4), IL-4 (clone BV6D-2462), and IFN-γ (clone XMG1.2), were obtained from eBioscience. Purified anti-mouse IL-4 (clone 11B11) and IFN-γ (clones AN18 and XMG1.2) were obtained from huvioma cell lines as previously described (22). Purified anti-mouse MIP-1α (clone 39624.11) and biotinylated anti-mouse MIP-1α (clone BAF450) were obtained from R&D Systems. The following FITC-, R-PE-, PE-Cy5.5-, PE-Cy7-, PerCP-, PerCP-Cy5.5-, allophycocyanin-, allophycocyanin-Cy7, and biotin-conjugated Abs were purchased from BD Pharmingen: anti-Ly-6A/E (sca-1) (clone D7), anti-CD73 (clone Y/23), anti-CD4, and biotin-conjugated Abs were purchased from BD Pharm-PE-Cy5.5-, PE-Cy7-, PerCP-, PerCP-Cy5.5-, allophycocyanin-, allophycocyanin-Cy7, and biotin-conjugated Abs were purchased from BD Pharmingen: anti-Ly-6A/E (sca-1) (clone D7), anti-CD73 (clone Y/23), anti-CD4, and biotin-conjugated Abs were purchased from BD Pharm-

Isolation of CD4 T cells

Splenocytes isolated from C57BL/6 mice were evaluated for IL-2 secretion by the Miltenyi cytokine secretion assay (Miltenyi Biotec) according to the manufacturer’s protocol. Twenty million splenocytes were stimulated with anti-CD3 (2 μg/ml) and anti-CD28 (1 μg/ml) per well in a 12-well culture plate for 12 h. Cells were collected and labeled with the IL-2 capture reagent followed by a 45-min secretion period, and subsequent staining with the PE-conjugated anti-IL2 detection Ab 7AAD and anti-CD4 and anti-CD19 Abs. FoxP3 expression was then determined as indicated above.

Cytokine detection

Detection of IL-2, IFN-γ, and MIP-1α by ELISPOT assay was performed as previously described (22). Detection of cytokines by Fluorospot assay was performed as previously described (32). Briefly, detection of IFN-γ and IL-4 by Fluorospot was performed by coating with either 2 μg/ml anti-IFN-γ Ab (clone AN18) or 10 μg/ml anti-IL-4 (11B11) with 2 μg/ml anti-CD3 and 1 μg/ml anti-CD28. Anti-IFN-γ (clone XMG1.2) or anti-IL-4 biotinylated Ab was added at 1 μg/ml. After incubation, the plates were washed in PBS containing 0.1% Tween 20 (PBST) and 2 μg/ml streptavidin-conjugated Cy3 in PBST plus 2% BSA was added for 30 min. The plates were washed again with PBST, dried, and scanned using a fluorescent microscope, and the digitized images were quantified by the ExploraSpot image analysis program (J. Rebhahn, J. J. Kobie, D. M. Zais, T. R. Mosmann, results not shown).

Coculture of sorted cells with Th1 cells

Naive, Treg, or Thpp cell populations were isolated as described above and cultured in 96-well round-bottom plates in X-VIVO 20 medium (Cambrex) in the presence of 0.5 μM 5′-AMP (Sigma-Aldrich) and 100 μM α,β-methylene ADP (APCP) (Sigma-Aldrich) or 25 μM SCH58261 (Sigma-Aldrich) as indicated for 4 h at 37°C. After addition of Th1 cells, IFN-γ secretion was measured by restimulation in 4-h Fluorospot assays as described above.

Statistical analysis

Where statistical difference is indicated two-tailed paired Student’s t tests with a 95% confidence interval were performed using Prism software (GraphPad).

Results

Expression of CD73 by distinct uncommitted primed precursor and regulatory T cell populations

Uncommitted precursor Thpp-like CD4 T cells (secreting IL-2 and chemokines) are mainly CD4^+ CD73^+ Ly-6A/E^− (22), whereas naive (CD4^low) CD4 T cells (secreting only IL-2) do not express significant levels of either CD73 or Ly-6A/E. Effector cells (secreting IFN-γ or IL-4) express high levels of Ly-6A/E in Ly-6.2 (C57BL/6, C57L, PL, C58, SJL, and AKR) mouse strains, but most effectors do not significantly express CD73 (22). Fig. 1a shows that most CD25^+ CD4^+ T cells from the spleens of C57BL/6 mice also expressed CD73, raising the possibility that Treg and Thpp cells also express CD73. Although Treg cells also express CD73, raising the possibility that Treg cells also express CD73. Although Treg cells also express CD73.
Both Thpp-like and CD25+ FoxP3+ cells expressed CD73 directly ex vivo, and the in vitro derivation of both Thpp and Treg cells from naive CD4 T cell populations can be enhanced by TGF-β (24, 33). Despite these similarities, the lack of expression of CD25 on most Thpp-like cells ex vivo (34) suggested that Thpp and Treg cells are separate populations, defined mainly by the surface marker patterns CD73+Ly-6A/E+/CD25−FoxP3− (Thpp) and CD73−Ly-6A/E−/CD25+FoxP3+ (Treg). Two functional properties of Thpp and Treg cells were used to test this hypothesis: Thpp cells secrete IL-2 on stimulation, and Treg cells suppress T cell responses.

CD25−CD73+Ly-6A/E− (potential Thpp) and CD25+CD73+ (potential Treg) populations were isolated by cell sorting from splenocytes, stimulated through the TCR, and their cytokine production profiles were tested by an ELISPOT assay. The putative Thpp-enriched population produced IL-2 and MIP-1α but not IFN-γ, consistent with the Thpp cytokine profile (35). In contrast, negligible numbers of the putative Treg population produced IL-2 or IFN-γ, and only a low frequency produced MIP-1α (Fig. 2a).

Naive (CD25−CD44low) cells produced primarily IL-2 (Fig. 2a). Although these results strongly suggested that Treg and Thpp cells were separate populations, a minority of FoxP3+ cells are CD25+ and therefore are present in the putative Thpp population (data not shown). To exclude the possibility that these cells were contributing to IL-2 production, IL-2 secretion and FoxP3 expression were measured by flow cytometry in splenocytes following anti-TCR stimulation. As the fixation methods for intracellular cytokine staining and FoxP3 expression were incompatible, the Miltenyi cytokine secretion assay was used to detect IL-2-producing cells. The FoxP3+ and IL-2+ splenocyte cell populations were separate, indicating that FoxP3+ cells within the CD4+CD25−CD73−Ly-6A/E− population do not contribute to the observed IL-2 production (Fig. 2b).

A subpopulation of FoxP3+ cells was also present in cultures of cells differentiated in vitro under Thpp-polarizing conditions (TGF-β and anti-IFN-γ), raising the possibility that the “Thpp” cultures also contained some Treg cells. This was supported by the observation that FoxP3+ cells were also distinct from IL-2-secreting cells in Thpp populations derived in vitro (Fig. 2b) as in the ex vivo populations. Similar IL-2 and FoxP3 expression patterns were observed following the stimulation of splenocytes and Thpp cells with alloantigen or staphylococcal enterotoxin B (data not shown). As expected, cells differentiated in vitro in Th1-polarizing conditions (IL-12) did not contain a FoxP3+ population (Fig. 2b).

We next tested whether the functions of ex vivo Thpp (CD25−CD73−Ly-6A/E−) and Treg (CD25+CD73+Ly-6A/E+) cells matched their FoxP3+ and cytokine expression patterns. Suppression of lymphocyte proliferation was measured by soluble anti-CD3 stimulation of CFSE-labeled splenocytes in the presence of sorted Thpp, Treg, or naive cells (and the absence of the CD73 substrate 5′-AMP). Treg cells markedly suppressed proliferation of CD4+ and CD8+ T cells (>50% of the cells remained undivided); however, naive or Treg cells had minimal effects on target cell proliferation (Fig. 3). When Thpp cells were present at the highest amount (1:2), a slight inhibition of CD4+ proliferation was observed that may have been a result of the FoxP3+ cells present in the Thpp population. No significant difference in suppressive activity was observed between CD25−CD73−Ly-6A/E− and CD25+CD73−Ly-6A/E− cells (data not shown).

Together, these results demonstrate that although CD73 is expressed by both Thpp and Treg populations, each has distinct characteristics; only the Thpp cells secrete IL-2, and only the Treg population is capable of suppression in the anti-CD3-stimulated splenocyte proliferation assay.

CD73 expression by Treg cells is consistent with their overall repertoire of suppressive functions, as CD73 generates adenosine, which suppresses some T cell effector functions. Expression of CD73 (and hence adenosine) may also be consistent with Treg cell functions, as Treg may represent a restrained T cell response that allows Ag-specific T cell expansion without strong, potentially harmful effector functions. Thus, Treg may not only lack strong effector functions but may also dampen the effector functions of Th1 or Th2 cells in the vicinity. To determine whether the amount of CD73 expressed by Treg and Thpp populations could convert sufficient 5′-AMP to adenosine to suppress Th1 and Th2 responses, we first determined the sensitivity of Th1 and Th2 responses to adenosine and then analyzed the suppression of Th1 responses by adenosine generated by Thpp or Treg cells.

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**FIGURE 1.** CD73 is expressed by T regulatory and Thpp cells. Splenocytes from C57BL/6 mice were isolated and analyzed by flow cytometry for cell surface marker expression (a and b) and intracellular expression of FoxP3 (b). Data are representative of at least three independent experiments.
Adenosine inhibits the cytokine production and proliferation of Th1 and Th2 cells

Adenosine has been demonstrated to inhibit T cell production of IFN-\(\gamma\) (13), although this required concentrations (50 \(\mu\)M) (13, 36) that were considerably higher than the \(K_d\) of the A2A receptor for various adenosine analogues (16). The use of hydrolysis-resistant A2A receptor agonists and A2A receptor antagonists has demonstrated that suppression of IFN-\(\gamma\) production by effector CD4 T cells is mediated through the A2A receptor (15). In preliminary experiments we confirmed that IFN-\(\gamma\) synthesis by Th1 cells was inhibited only at high adenosine concentrations, and so we addressed the possibility that high concentrations were required because adenosine was rapidly degraded in culture. Th1 cells, prepared by in vitro differentiation (35), were stimulated with anti-CD3 and anti-CD28 Abs in the presence of varying concentrations of adenosine, and the number of IFN-\(\gamma\)-secreting cells was measured by a Fluorospot assay (32). In medium containing serum, adenosine minimally reduced the number of IFN-\(\gamma\)-secreting cells even at a concentration of 10 \(\mu\)M (data not shown), whereas in serum-free medium even 0.1 \(\mu\)M adenosine significantly \((p < 0.0001)\) inhibited IFN-\(\gamma\) production (Fig. 4). The sensitivity of the Th1 cells to suppression by <0.1 \(\mu\)M adenosine in serum-free medium (Fig. 5) is consistent with the \(K_d\) of the A2A receptor for several adenosine analogues (16). Serum did not significantly alter
CD73 MEDIATES Thpp AND Treg SUPPRESSION OF Th1/Th2 RESPONSES

FIGURE 4. Adenosine inhibits the cytokine production and proliferation of Th1 and Th2 cells. a, Th1 and Th2 cells were cultured in X-VIVO 20 medium with the absence or presence of 1 μM adenosine or 5’-AMP and stimulated with plate-bound anti-CD3 and anti-CD28 in either IFN-γ or IL-4 Fluorospot assays for 4 and 16 h, respectively. Data are representative of mean ± SEM of triplicate wells. *p < 0.01 (significant difference). b, CFSE-labeled Th1 or Th2 cells were stimulated in X-VIVO 20 medium with plate-bound anti-CD3 and anti-CD28 with IL-2 in the presence of 10 μM adenosine for 72 h and stained with 7AAD, anti-CD4, and anti-CD19 and evaluated for proliferation by flow cytometry. Data are mean ± SD of triplicate wells. *p < 0.05 (significant difference).

FIGURE 5. Suppression of IFN-γ production by adenosine is mediated through the A2a receptor. Th1 cells were stimulated in IFN-γ Fluorospot assays as indicated above with increasing doses of adenosine or 5’-AMP in the presence of the A1 receptor antagonist 1,3-dipropyl-8-cyclopentylxanthine (DP-CPX; 5 μM) or the A2a receptor antagonist SCH58261 (5 μM). Means ± SEM of triplicate wells are shown. Data are representative of three independent experiments.

The effects of adenosine on proliferation of Th1 and Th2 cells were tested by stimulating CFSE-labeled Th1 or Th2 cells with anti-CD3, anti-CD28, and IL-2 in serum-free medium in the presence or absence of adenosine. Proliferation of Th1 or Th2 cells, as measured by dilution of CFSE, was significantly (p = 0.0006 and 0.0003, respectively) inhibited by adenosine (Fig. 4a).

Consistent with previous reports, the suppressive activity of adenosine on Th1 cytokine secretion was abrogated by the addition of the A2A receptor antagonist SCH58261(14), whereas the A1 receptor antagonist 1,3-dipropyl-8-cyclopentylxanthine (37) had minimal effects on the suppressive activity (Fig. 4b).

Coculture of Treg and Thpp with Th1 cells in the presence of 5’-AMP suppresses IFN-γ production

The suppression of T cell cytokine production by even low concentrations of adenosine raised the possibility that the CD73 expressed on Thpp and Treg cells could generate sufficient adenosine (by cleaving 5’-AMP) to inhibit effector T cell function. This was tested by coculturing Th1 cells with ex vivo isolated Thpp (CD25−CD73+Ly-6A/E−), Treg (CD25+CD73−), or naive (CD73−CD25−CD44−) CD4 T cells that had been preincubated with physiological dilutions of 5’-AMP (38) and measuring the number of Th1 cells secreting IFN-γ in response to anti-CD3 and anti-CD28 stimulation. In the absence of 5’-AMP, none of these three T cell populations inhibited the synthesis of IFN-γ by anti-CD3- and anti-CD28-stimulated Th1 cells (Fig. 6). The lack of Treg-mediated suppression of IFN-γ synthesis in the absence of 5’-AMP is consistent with previous reports indicating that stimulation through CD28 can block Treg-mediated suppression (39–41). However, the presence of 5’-AMP at concentrations as low as 0.5 μM resulted in the inhibition of Th1 IFN-γ synthesis in cultures containing CD73+ Thpp or Treg cells, but not CD73− naïve cells. The role of CD73 in inhibiting IFN-γ production was confirmed by showing that suppression was prevented by a specific inhibitor of CD73 enzymatic activity, ACP (42). An A2A receptor antagonist (SCH58261) also prevented suppression, confirming that the effect was mediated through adenosine (Fig. 6). The adenosine-mediated inhibitory effect of Thpp and Treg cells was exerted at ratios of less than 1:1 of suppressor to target cells.

Discussion

Taken together, these results strongly support a model (Fig. 7) in which the expression of CD73 by the two distinct CD4 T cell subsets, Thpp and Treg, confers a potential anti-inflammatory function. In this model, 5’-AMP released at a site of ongoing inflammation (43, 44) is cleaved by CD73 on local Treg or Thpp cells, and the resulting adenosine acts directly through the A2A receptor on effector T cells such as Th1 cells to inhibit their proliferation and secretion of cytokines. This in turn will mitigate ongoing inflammation, providing negative feedback regulation between cell damage and release of 5’-AMP) and inflammatory cytokine production.

This potential suppressive pathway is distinct from the conventional suppressive mechanism of Treg cells, as revealed in different assays (Figs. 3 and 6). If target T cells are stimulated only with anti-CD3 (without anti-CD28 or 5’-AMP), the resulting proliferation can be suppressed by Treg (but not Thpp) cells. In this conventional Treg assay, costimulation with anti-CD28 Abs prevents suppression by Tregs. However, even in the presence of both anti-CD3 and anti-CD28, Treg and Thpp cells can suppress by the CD73-dependent pathway if the substrate 5’-AMP is added.

Because both Thpp and Treg cells can be found in peripheral tissue (22, 26, 45, 46), it is likely that, at an inflammatory site, adenosine generated by these cell types could contribute to other anti-inflammatory activities including suppression of B cell, neutrophil, monocyte, macrophage, and dendritic cell functions (3). Adenosine-mediated suppression of IFN-γ production occurred at a ratio of less
than one Thpp or Treg per effector T cell, suggesting that this could be an effective regulatory mechanism of inflammatory reactions.

Even after more than a decade of heightened attention, the mechanism(s) by which Tregs mediate suppression of immune responses remains unclear. In particular, the relationship between their activity in conventional in vitro suppressor assays and their responses remains unclear. In particular, the relationship between the mechanism(s) by which Tregs mediate suppression of immune responses, without the risk of future differentiation into effector cells.

**References**


